

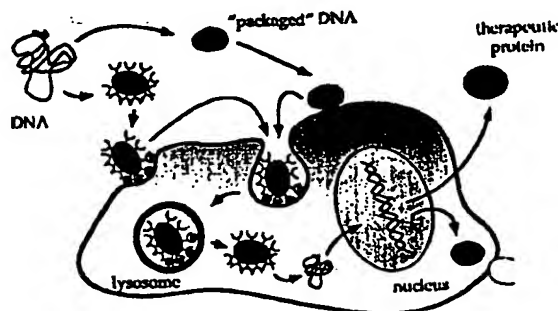


INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/00	A2	(11) International Publication Number: WO 99/42091
		(43) International Publication Date: 26 August 1999 (26.08.99)
<p>(21) International Application Number: PCT/US99/03294</p> <p>(22) International Filing Date: 16 February 1999 (16.02.99)</p> <p>(30) Priority Data: 60/075,272 19 February 1998 (19.02.98) US</p> <p>(71) Applicant: MASSACHUSETTS INSTITUTE OF TECHNOLOGY [US/US]; 77 Massachusetts Avenue, Cambridge, MA 02139 (US).</p> <p>(72) Inventors: LANGER, Robert, S.; 77 Lombard Street, Newton, MA 02158 (US). PUTNAM, David, A.; 143 Otis Street, Cambridge, MA 02141 (US). PACK, Daniel, W.; 3105 Goldmedal Drive, Campagne, IL 61822 (US).</p> <p>(74) Agent: SHAIR, Karoline, K., M.; Choate, Hall & Stewart, Exchange Place, 53 State Street, Boston, MA 02109 (US).</p>		<p>(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>

(54) Title: **CELL DELIVERY COMPOSITIONS**

Gene delivery vehicles transport DNA across the cell membrane and into the cytoplasm.



(57) Abstract

The present invention provides improved cell delivery compositions. In particular, the invention provides biocompatible endosomolytic agents. In a preferred embodiment, the endosomolytic agents are also biodegradable and can be broken down within cells into components that the cells can either reuse or dispose of. Preferred endosomolytic agents include cationic polymers, particularly those comprised of biomolecules, such as histidine, polyhistidine, polylysine or any combination thereof. Other exemplary endosomolytic agents include, but are not limited to, other imidazole containing compounds such as vinylimidazole and histamine. More particularly preferred are those agents having multiple proton acceptor sites and acting as a "proton sponge", disrupting the endosome by osmolytic action. In preferred embodiments, the endosomolytic agent comprises a plurality of proton acceptor sites having pKas within the range of 4 to 7, which endosomal lysing component is polycationic at pH 4. The present invention also contemplates the use of these endosomolytic agents as delivery agents by complexation with the desired compound to be delivered. Thus, the present invention also acts as a cell delivery system comprising an endosomolytic agent, a delivery agent, and a compound to be delivered.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
				PT	Portugal		

CELL DELIVERY COMPOSITIONS

Priority Information

5 This application claims priority to the co-pending provisional application number 60/075,272 entitled "Cell Delivery Compositions" filed on February 19, 1998, which is incorporated in its entirety by reference.

Government Support

5 The present research was supported by a grant from the National Institutes of Health (Grant Number GM26698).

Background of the Invention

10 The recent revolutions in molecular and pharmaceutical biology and pharmaceutical chemistry have created a need for the development of effective mechanisms for delivering biological and other therapeutic agents into cells. Researchers have particularly struggled to develop an efficient means of introducing nucleic acids into
15 cells, for example for gene therapy, antisense therapy, or research purposes (e.g., to study cell differentiation, growth and carcinogenic transformation or to create animal models for human disease; see, for example, Abdallah, *Biol. Cell*, 85:1, 1995 and references therein).

Unfortunately, existing techniques for delivering nucleic acids to cells are limited by poor efficiency and/or high toxicity of the delivery reagents. A particular
20 problem is encountered with techniques that rely on receptor-mediated endocytosis (see, e.g., Figure 1) because the nucleic acid to be delivered is often destroyed when exposed to the low pH and active degradatory machinery of the endosome/lysosome. Various reagents (e.g., chloroquine, polyethylenimine [PEI], certain highly charged cationic compounds, fusogenic peptides, and inactivated adenoviruses) have been developed that
25 are intended to quickly disrupt the endosome in order to minimize the amount of time that a delivered nucleic acid spends in this hostile environment.

Certain of these known compounds (i.e., chloroquine, PEI), are thought to act as so-called "proton-sponges" because they contain a large number of proton-acceptor

5 sites. It is thought that these compounds sop up protons in the endosome, thereby increasing the pH in the endosome (see, for example, Boussif et al., *PNAS*, 92:7297, 1995). This pH increase both inhibits the action of lysosomal nucleases with acid-optimal pH dependence and induces an ATPase proton pump in the endosomal membrane to furiously pump additional protons from the cytoplasm into the endosome
10 in order to restore the proper endosomal pH. Because the ATPase pump carries one chloride ion into the endosome with every proton that it transfers from the cytoplasm, its excessive pumping creates an osmotic pressure imbalance that results in lysis of the endosome (see Behr, ILMAC, 1st Swiss Cost Chemistry Symposium, 1996; see also Figure 2).

15 The highly charged cationic compounds are thought to burst open the endosomal compartment by a different mechanism that involves fusing with and lysing open the bilayer membranes. The fusogenic peptides and inactivated viruses rely on viral lysis capabilities to burst the endosome compartment.

Although these known endosomolytic agents do appear to increase the
20 efficiency of nucleic acid delivery, they have serious toxicity problems and other disadvantages. Some (e.g., chloroquine) are simply poisonous to cells. Others (e.g., viral compounds) can activate the immune system, thereby risking systemic difficulties and also creating the possibility that the host immune system will destroy the agent relied upon to effect cell delivery. There remains a need for the development of a
25 biocompatible, preferably biodegradable, endosomolytic cell delivery agent. There is a particular need for an agent that can efficiently introduce nucleic acids into cells.

Summary of the Invention

The present invention provides improved cell delivery compositions. In particular,
30 the invention provides a biocompatible endosomolytic system. These inventive endosomolytic agents obviate the need for known agents (i.e., chloroquine, fusogenic peptides, inactivated adenoviruses, and polyethyleneimine) that can burst endosomes but have negative effects on cells. Preferred inventive endosomolytic agents are biodegradable in that they are broken down within cells into components that the cells can
35 either reuse or dispose of. Particularly preferred inventive endosomolytic agents are cationic polymers comprised of biomolecules. Although the present invention is not

5 limited by the mechanism of action of the endosomolytic agents, certain preferred agents have multiple proton acceptor sites and would be expected to act as "proton sponges", disrupting the endosome by osmolytic action. Particularly preferred agents are polycationic under the conditions of the endosome (i.e., at pH 4). Exemplary endosomolytic agents include, but are not limited to, imidazole containing compounds
10 such as histidine, histamine, vinylimidazole, polymers thereof, and any combinations thereof.

In one preferred embodiment of the invention, the endosomolytic agent comprises polyhistidine. Polyhistidine for use in accordance with the present invention may be provided as a linear or branched polyhistidine polymer. Moreover, as is discussed further
15 below, the polyhistidine may be provided in combination with one or more additional agents. Where such other agents are other polymers, or functionalizable chemical compounds, they may be co-polymerized or functionalized with polyhistidine or histidine. Thus, a polyhistidine endosomolytic agent of the present invention need not comprise a polyhistidine polymer *per se*, so long as it has a sufficient number of histidine functional
20 groups to preserve polyhistidine functionality as described herein. To give but one example, the inventive endosomolytic agent may comprise a single linear or branched copolymer synthesized from any appropriate combination of polyhistidine, polylysine, histidine, and/or lysine.

The endosomolytic agents of the present invention may be employed in any of a
25 variety of delivery contexts. In some cases, the endosomolytic agent also acts as a delivery agent; in other cases, the endosomolytic agent is combined with a delivery agent that complexes the compound being delivered in a manner that allows that compound to be taken into an endosome and thereby introduced into a cell. Thus, the present invention also provides a cell delivery system comprising an endosomolytic agent, a delivery agent,
30 and a compound to be delivered. In preferred embodiments, the compound to be delivered comprises nucleic acid. Also, certain preferred cell delivery systems include a targeting agent, preferably covalently linked to one or more of the endosomolytic agent, the delivery agent, and the delivery compound.

In one particularly preferred embodiment of the cell delivery system of the present
35 invention, the endosomolytic agent comprises or consists of polyhistidine, the delivery agent comprises or consists of polylysine, and the delivery compound comprises or

5 consists of nucleic acid, preferably DNA. The polyhistidine and polylysine may be mixed together as separate components or may be formulated together as a single linear or branched copolymer. That is, any appropriate combination of polyhistidine and polylysine, polyhistidine and lysine, or histidine and polylysine may be employed in accordance with the present invention.

10

Definitions

"Biocompatible"-- The term "biocompatible", as used herein is intended to describe compounds that are not toxic to cells. Compounds are "biocompatible" if their addition to cells *in vitro* results in less than or equal to 20 % cell death and do not induce inflammation or other such adverse effects *in vivo*.

15

"Biodegradable"-- As used herein, "biodegradable" compounds are those that, when introduced into cells, are broken down by the cellular machinery into components that the cells can either reuse or dispose of without significant toxic effect on the cells (i.e., fewer than about 20 % of the cells are killed).

20

"Biomolecules"-- The term "biomolecules", as used herein, refers to molecules (e.g., proteins, amino acids, nucleic acids, nucleotides, carbohydrates, sugars, lipids, etc.) that are found in living cells in nature.

25

"Known endosomolytic agents"-- The phrase "known endosomolytic agents", as used herein, refers to a particular set of compounds: chloroquine, fusogenic peptides, inactivated adenoviruses, and polyethyleneimine, that were known on the day the present application was filed to have osmolytic capabilities. The classification of such compounds as "known" is not intended to represent that such compounds constitute prior art to the present invention, nor is it intended to represent that the osmolytic capability of the agent was known prior to the date of the present invention.

30

"Proton sponge"-- The term "proton sponge", as used herein, refers to a compound with a sufficient number of proton acceptor sites that, when the compound is introduced into an endosome within a living cell, endosomal protons associate with the compound so that the endosomal pH rises, the endosomal proton pump is activated to transfer protons and counter ions into the endosome, and the osmotic pressure within the endosome rises to a point that bursts the endosome. "Proton sponge" is used interchangeably with "osmolytic agent" herein.

35

Description of the Drawing

5

Figure 1 diagrams delivery of DNA into a cell cytoplasm by receptor mediated endocytosis.

Figure 2 depicts the process by which "proton-sponge" polymers are believed to mediate release of delivered compounds (e.g., DNA) from lysosomes.

10

Figure 3 depicts of the chemical structure of polyhistidine.

Figure 4 shows the chemical structure of polyhistidine and its protonation as a function of pH demonstrating that polyhistidine functions as a biopolymeric proton sponge.

15

Figures 5A and 5B depict certain preferred cell delivery compositions of the present invention. In particular, Figure 5A shows a copolymer of polylysine and histidine. Figure 5B shows a copolymer of polylysine and polyhistidine.

Figure 6 is a schematic showing derivatization of polyhistidine with gluconic acid that results in improved solubility of gluconoyl-polyhistidine at neutral pH.

20

Figure 7 is a graph demonstrating that DNA/gluconoyl-polyhistidine/transferrin-polylysine complexes are well below the size limit of 150 nm for endocytosis.

Figure 8 shows that DNA/gluconoyl-polyhistidine/transferrin-polylysine complexes transfect approximately 10% of COS-7 cells by X-gel staining.

Figure 9 is a graph that shows the DNA/gluconoyl-polyhistidine/transferrin-polylysine complexes effectively transfect COS-7 cells in culture.

25

Figure 10 is a graph showing that gluconoyl-polyhistidine is non-toxic to COS-7 cells *in vitro*.

Figure 11 is a graph that represents data from ethidium bromide exclusion assays showing gluconoyl-polyhistidine condenses plasmid DNA efficiently at pH 5, but plasmid DNA is less condensed at pH 7.4.

30

Figure 12 is a graph showing ethidium exclusion by DNA/transferrin-polylysine and DNA/gluconoyl-polyhistidine/transferrin-polylysine complexes.

Figure 13 presents gel electrophoresis of DNA/transferrin-polylysine mixtures that reveals complex formation.

35

Figure 14 presents gel electrophoresis of DNA/gluconoyl-polyhistidine mixtures that reveals complex formation.

5 Figure 15 presents gel electrophoresis of DNA/polyhistidine mixtures revealing complex formation.

Detailed Description of the Preferred Embodiments

10 In recognition of the importance of the development of a safe and effective cell delivery system, the present invention provides improved compositions and methods for the delivery of therapeutic agents to cells and subcellular components. In one aspect, the present invention provides a biocompatible endosomolytic system. These inventive endosomolytic agents obviate the need for known agents (i.e., chloroquine, fusogenic peptides, inactivated adenoviruses, and polyethyleneimine) that can burst endosomes but
15 have negative effects on cells. In another aspect, the present invention provides a cell delivery system comprising an inventive endosomolytic agent, and a delivery agent. Certain examples of preferred endosomolytic systems and cell delivery systems are presented below.

20 *Endosomolytic agents*

 As discussed above, the present invention provides an improved system for delivery of compounds to cells and lysis of endosomal cell compartments. In particular, the invention provides biocompatible, preferably biodegradable, endosomolytic agents. While the mechanism of action of the endosomolytic agents is not intended to limit the
25 scope of the present invention, preferred agents have multiple proton acceptor sites (i.e., multiple groups with a pKa intermediate between pH 4 and pH 7) and/or are polycationic, at least when they are within the endosome. Particularly preferred agents are linear or branched polymers of biomolecules, preferably of amino acids or amino acid derivatives. Exemplary endosomolytic agents include, but are not limited to, imidazole containing
30 compounds such as histidine, histamine, vinylimidazole, polymers thereof, and any combinations thereof.

 As one of ordinary skill in the art will realize, the endosomolytic agents of the present invention must be of appropriate size to fit inside an endosomal compartment, along with any agent to be delivered to the cell. Inventive agents are therefore less than
35 about 150 nm in size, or are capable of adopting a conformation less than about 150 nm in size for purposes of uptake via endocytosis.

5 Polyhistidine (Figure 3) is one example of a particularly preferred endosomolytic agent of the present invention. The histidine imidazole side chain has a pKa of 6.5, so that polyhistidine has multiple proton acceptor sites according to the present invention. Polyhistidine is protonated, and therefore polycationic, at pH 4 (i.e., within the endosome). As shown in Figure 4, polyhistidine is expected to act as a proton sponge in
10 endosomes; the present invention is not limited to such a mechanism, however.

The polyhistidine endosomolytic agent of the present invention may be a linear polymer or a branched polymer. Moreover, the polyhistidine may be combined or polymerized with one or more additional agents with desirable cell delivery attributes. For example, the polyhistidine may be combined with a delivery agent selected to interact
15 with the compound to be delivered to the cell. However, the polyhistidine of the present invention is not combined with chloroquine, fusogenic peptides, inactivated adenoviruses, and polyethyleneimine.

In another particularly preferred embodiment of the present invention, polyhistidine is combined with polylysine to deliver nucleic acid to cells. Polylysine is
20 known to bind to nucleic acids and to compact them (Cotten et al., *Methods Enz.*, 217: 644, 1993). Thus, polylysine is a useful delivery agent for nucleic acids. In fact, prior to the present invention, efforts had been made to use polylysine for delivery of nucleic acids to cells (see, for example, Wagner et al., *PNAS*, 87: 3410, 1990). However, polylysine-mediated delivery was inefficient in the absence of an endosomolytic agent, and known
25 endosomolytic agents were toxic. The present invention remedies this difficulty.

Figures 5A and 5B depict certain polyhistidine and polyhistidine/polylysine compositions of the present invention. Polyhistidine and polylysine can be prepared (or purchased) separately and combined together in various ratios; or can be covalently linked
30 to one another in linear or branched co-polymers of any form (e.g., graft co-polymers, dendritic co-polymers, etc.). Moreover, histidine and lysine may be "polymerized together", such that the product polymer contains histidine and lysine units in any desirable arrangement.

Those of ordinary skill in the art will, using known techniques, be able to prepare any of a variety of polyhistidine/polylysine compositions that can readily be tested
35 according to the teachings herein to identify those with desirable delivery characteristics. The compositions must have sufficient polyhistidine composition (including available

5 proton acceptor sites and/or polycationic character) to lyse endosomes, and sufficient polylysine composition to bind to nucleic acids, and condense them if necessary. Thus, the inventive polyhistidine/polylysine composition may comprise any combination of polylysine with polyhistidine, polylysine with histidine, or lysine with polyhistidine, associated with one another covalently or otherwise, so long as the combination is
10 biocompatible and has the endosomolytic and nucleic acid binding/packaging capabilities described herein. As one of ordinary skill in the art will realize, the entire composition (including the bound nucleic acid) must be small enough to be taken up into cells. As mentioned above, endosomal compartments can usually accept entities up to about 150 nm in size.

15 In addition, or as an alternative to being combined with a delivery agent, the endosomolytic agent of the present invention may be combined with one or more other agents to achieve, for example, a desired solubility or targeting to a particular cell or cell type. Cell targeting is discussed in more detail below; solubility adjustments are readily accomplished, for example, by functionalizing the endosomolytic agent, or another factor
20 with which it is associated, with a hydrophilic moiety. For example, the above-described polyhistidine endosomolytic agent of the present invention can be solubilized through functionalization with gluconic acid (see Figure 6) or other moieties including but not limited to, carbohydrates, nucleic acids, and amino acids.

25 *Delivery agents*

As one of ordinary skill in the art will realize, the selection of delivery agent for use in accordance with the present invention depends on the compound to be delivered. The delivery agent is thus any biocompatible (preferably biodegradable) entity that interacts with the compound to be delivered in such a way as to mediate its introduction
30 into a cell.

For example, as discussed above, polylysine is a useful delivery agent for nucleic acids. Other nucleic acid delivery agents can readily be identified. For example, compounds with a high charge density are likely to be able to interact with, and often package, DNA. Preferred compounds are biopolymers (i.e., polymers of biomolecules)
35 with at least one charge per monomer unit.

5 Those of ordinary skill in the art will recognize that inventive compositions comprising an endosomolytic agent, a nucleic acid delivery agent, and a nucleic acid are, in effect, artificial viruses characterized in being non-immunogenic, capable of circulation in the bloodstream, targetable to particular cells (i.e., when a targeting agent is employed) and less than 150 nm in size.

10 *Targeting agents*

It is often desirable to target a cell delivery composition to a particular cell or collection of cells. A variety of agents that direct compositions to particular cells are known in the art (see, for example, Cotten et al., *Methods Enzym*, 217: 618, 1993). Preferred targeting agents are biocompounds, or portions thereof, that interact specifically with individual cells, small groups of cells, or large categories of cells. Examples of useful targeting agents include, but are in no way limited to, low-density lipoproteins (LDLs), transferrin, asialoglycoproteins, gp120 envelope protein of the human immunodeficiency virus (HIV), and diphtheria toxin, antibodies, and carbohydrates.

15 Certain preferred endosomolytic compositions of the present invention include one or more targeting agents associated with (e.g., by covalent, hydrophobic, hydrogen-bonding, van der Waals, or other interaction) the inventive endosomolytic agent, the delivery agent, and/or the delivery compound. To give but one example, Example 2 describes a polyhistidine/polylysine inventive composition in which at least some of the polylysine is covalently linked to transferrin. As shown in Figure 7, this composition is less than 150 nm in size.

20 *Delivery compounds*

In principle, any substance having biological activity may be delivered to cells using the endosomolytic and/or cell delivery systems of the present invention. For example, the invention includes but is not limited to delivery of proteins, polypeptides, polynucleotides, nucleoproteins, polysaccharides, glycoproteins, lipoproteins, and synthetic and biologically engineered analogs thereof.

25 Examples of biologically active compounds that might be utilized in a delivery application of the invention include literally any hydrophilic or hydrophobic biologically active compound. Preferably, though not necessarily, the drug is one that

5 has already been deemed safe and effective for use by the appropriate governmental agency or body. For example, drugs for human use listed by the FDA under 21 C.F.R. §§ 330.5, 331 through 361; 440-460; drugs for veterinary use listed by the FDA under 21 C.F.R. §§ 500-582, incorporated herein by reference, are all considered acceptable for use in the present inventive cell delivery composition.

10 Biologically active compounds for use in the present invention include any pharmacologically active substances that produce a local or systemic effect in animals, preferably mammals, or humans. The term thus means any substance intended for use in the diagnosis, cure, mitigation, treatment or prevention of disease or in the enhancement of desirable physical or mental development and conditions in an animal
15 or human.

Classes of pharmaceutically active compounds that can be used in the practice of the present invention include, but are not limited to, anti-AIDS substances, anti-cancer substances, antibiotics, immunosuppressants (e.g., cyclosporine), anti-viral substances, enzyme inhibitors, neurotoxins, opioids, hypnotics, antihistamines, lubricants,
20 tranquilizers, anti-convulsants, muscle relaxants and anti-Parkinson substances, anti-spasmodics and muscle contractants, miotics and anti-cholinergics, anti-glaucoma compounds, anti-parasite and/or anti-protozoal compounds, anti-hypertensives, analgesics, anti-pyretics and anti-inflammatory agents such as NSAIDs, local anesthetics, ophthalmics, prostaglandins, anti-depressants, anti-psychotic substances,
25 anti-emetics, imaging agents, specific targeting agents, neurotransmitters, proteins, cell response modifiers, vaccines, ribozymes, anti-sense agents, and RNA.

A more complete listing of classes of compounds suitable for delivery into cells according to the present invention may be found in the Pharmazeutische Wirkstoffe (Von Kleemann et al. (eds) Stuttgart/New York, 1987, incorporated herein by
30 reference). Examples of particular pharmaceutically active substances are presented below:

Anti-AIDS substances are substances used to treat or prevent Autoimmune Deficiency Syndrome (AIDS). Examples of such substances include, but are not limited to, CD4, 3'-azido-3'-deoxythymidine (AZT),
35 9-(2-hydroxyethoxymethyl)-guanine (acyclovir), phosphonoformic acid, 1-adamantanamine, peptide T, and 2',3' dideoxycytidine.

5 Anti-cancer substances are substances used to treat or prevent cancer. Examples of such substances include, but are not limited to, methotrexate, cisplatin, prednisone, hydroxyprogesterone, medroxyprogesterone acetate, megestrol acetate, diethylstilbestrol, testosterone propionate, fluoxymesterone, vinblastine, vincristine, vindesine, daunorubicin, doxorubicin, hydroxyurea, procarbazine, aminoglutethimide, mechlorethamine, cyclophosphamide, melphalan, uracil mustard, chlorambucil, busulfan, 10 carmustine, lomusline, dacarbazine (DTIC: dimethyltriazenomidazolecarboxamide), methotrexate, fluorouracil, 5-fluorouracil, cytarabine, cytosine arabinoside, mercaptopurine, 6-mercaptopurine, thioguanine.

15 Antibiotics are art recognized and are substances which inhibit the growth of or kill microorganisms. Antibiotics can be produced synthetically or by microorganisms. Examples of antibiotics include, but are not limited to, penicillin, tetracycline, chloramphenicol, minocycline, doxycycline, vanomycin, bacitracin, kanamycin, neomycin, gentamycin, erythromycin and cephalosporins.

20 Anti-viral agents are substances capable of destroying or suppressing the replication of viruses. Examples of anti-viral agents include, but are not limited to, α -methyl-P-adamantane methylamine, 1,-D-ribofuranosyl-1,2,4-triazole-3 carboxamide, 9-[2-hydroxy-ethoxy]methylguanine, adamantanamine, 5-iodo-2'-deoxyuridine, trifluorothymidine, interferon, and adenine arabinoside.

25 Enzyme inhibitors are substances which inhibit an enzymatic reaction. Examples of enzyme inhibitors include, but are not limited to, edrophonium chloride, N-methylphysostigmine, neostigmine bromide, physostigmine sulfate, tacrine HCl, tacrine, 1-hydroxy maleate, iodotubercidin, p-bromotetramisole, 10-(α -diethylaminopropionyl)- phenothiazine hydrochloride, calmidazolium chloride, hemicholinium-3, 3,5-dinitrocatechol, diacylglycerol kinase inhibitor I, diacylglycerol 30 kinase inhibitor II, 3-phenylpropargylamine, N₆-monomethyl-L-arginine acetate, carbidopa, 3-hydroxybenzylhydrazine HCl, hydralazine HCl, clorgyline HCl, deprenyl HCl, L(-)-, deprenyl HCl, D(+)-, hydroxylamine HCl, iproniazid phosphate, 6-MeO-tetrahydro-9H-pyrido-indole, nialamide, pargyline HCl, quinacrine HCl, semicarbazide HCl, tranlycypromine HCl, N,N-diethylaminoethyl-2,2-diphenylvalerate 35 hydrochloride, 3-isobutyl-1-methylxanthine, papaverine HCl, indomethacin, 2-cyclooctyl-2-hydroxyethylamine hydrochloride, 2,3-dichloro-a-methylbenzylamine

5 (DCMB), 8,9-dichloro-2,3,4,5-tetrahydro-1H-2-benzazepine hydrochloride, p-aminoglutethimide, p-aminoglutethimide tartrate, R(+)-, p-aminoglutethimide tartrate, S(-)-, 3-iodotyrosine, alpha-methyltyrosine, L-, alpha-methyltyrosine, D L-, acetazolamide, dichlorphenamide, 6-hydroxy-2-benzothiazolesulfonamide, and allopurinol.

10 Neurotoxins are substances which have a toxic effect on the nervous system, e.g. nerve cells. Neurotoxins include, but are not limited to, adrenergic neurotoxins, cholinergic neurotoxins, dopaminergic neurotoxins, and other neurotoxins. Examples of adrenergic neurotoxins include N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride. Examples of cholinergic neurotoxins include acetylcholine mustard
15 hydrochloride. Examples of dopaminergic neurotoxins include 6-hydroxydopamine HBr, 1-methyl-4-(2-methylphenyl)-1,2,3,6-tetrahydro-pyridine hydrochloride, 1-methyl-4-phenyl-2,3-dihydropyridinium perchlorate, N-methyl-4-phenyl-1,2,5,6-tetrahydropyridine HCl, 1-methyl-4-phenylpyridinium iodide.

Opioids are substances having opiate like effects that are not derived from
20 opium. Opioids include opioid agonists and opioid antagonists. Opioid agonists include, but are not limited to, codeine sulfate, fentanyl citrate, hydrocodone bitartrate, loperamide HCl, morphine sulfate, noscapine, norcodeine, normorphine, thebaine. Opioid antagonists include, but are not limited to, nor-binaltorphimine HCl, buprenorphine, chlornaltrexamine 2HCl, funaltrexamine HCl, nalbuphine HCl,
25 nalorphine HCl, naloxone HCl, naloxonazine, naltrexone HCl, and naltrindole HCl.

Hypnotics are substances which produce a hypnotic effect. Hypnotics include, but are not limited to, pentobarbital sodium, phenobarbital, secobarbital, thiopental and mixtures, thereof, heterocyclic hypnotics, dioxopiperidines, glutarimides, diethyl isovaleramide, a-bromoisovaleryl urea, urethanes and disulfanes.

30 Antihistamines are substances which competitively inhibit the effects of histamines. Examples include, but are not limited to, pyrrolamine, chlorpheniramine, tetrahydrazoline, and the like.

Lubricants are substances that increase the lubricity of the environment into which they are delivered. Examples of biologically active lubricants include, but are
35 not limited to, water and saline.

5 Tranquilizers are substances which provide a tranquilizing effect. Examples of tranquilizers include, but are not limited to, chlorpromazine, promazine, fluphenzaine, reserpine, deserpidine, and meprobamate.

 Anti-convulsants are substances which have an effect of preventing, reducing, or eliminating convulsions. Examples of such agents include, but are not limited to,
10 primidone, phenytoin, valproate, Chk and ethosuximide.

 Muscle relaxants and anti-Parkinson agents are agents which relax muscles or reduce or eliminate symptoms associated with Parkinson's disease. Examples of such agents include, but are not limited to, mephenesin, methocarbomal, cyclobenzaprine hydrochloride, trihexylphenidyl hydrochloride, levodopa/carbidopa, and biperiden.

15 Anti-spasmodics and muscle contractants are substances capable of preventing or relieving muscle spasms or contractions. Examples of such agents include, but are not limited to, atropine, scopolamine, oxyphenonium, and papaverine.

 Miotics and anti-cholinergics are compounds which cause bronchodilation. Examples include, but are not limited to, echothiophate, pilocarpine, physostigmine
20 salicylate, diisopropylfluorophosphate, epinephrine, neostigmine, carbachol, methacholine, bethanechol, and the like.

 Anti-glaucoma compounds include, but are not limited to, betaxalol, pilocarpine, timolol, timolol salts, and combinations of timolol, and/or its salts, with pilocarpine.

 Anti-parasitic, -protozoal and -fungals include, but are not limited to, ivermectin,
25 pyrimethamine, trisulfapyrimidine, clindamycin, amphotericin B, nystatin, flucytosine, natamycin, and miconazole.

 Anti-hypertensives are substances capable of counteracting high blood pressure. Examples of such substances include, but are not limited to, alpha-methyldopa and the pivaloyloxyethyl ester of alpha-methyldopa.

30 Analgesics are substances capable of preventing, reducing, or relieving pain. Examples of analgesics include, but are not limited to, morphine sulfate, codeine sulfate, meperidine, and nalorphine.

 Anti-pyretics are substances capable of relieving or reducing fever and anti-inflammatory agents are substances capable of counteracting or suppressing
35 inflammation. Examples of such agents include, but are not limited to, aspirin (salicylic acid), indomethacin, sodium indomethacin trihydrate, salicylamide, naproxen,

5 colchicine, fenoprofen, sulindac, diflunisal, diclofenac, indoprofen and sodium salicylamide.

Local anesthetics are substances which have an anesthetic effect in a localized region. Examples of such anesthetics include, but are not limited to, procaine, lidocaine, tetracaine and dibucaine.

10 Ophthalmics include diagnostic agents such as sodium fluorescein, rose bengal, methacholine, adrenaline, cocaine, and atropine. Ophthalmic surgical additives include, but are not limited to, alpha-chymotrypsin and hyaluronidase.

Prostaglandins are art recognized and are a class of naturally occurring chemically related, long-chain hydroxy fatty acids that have a variety of biological effects.

15 Anti-depressants are substances capable of preventing or relieving depression. Examples of anti-depressants include, but are not limited to, imipramine, amitriptyline, nortriptyline, protriptyline, desipramine, amoxapine, doxepin, maprotiline, tranlycypromine, phenelzine, and isocarboxazide.

20 Anti-psychotic substances are substances which modify psychotic behavior. Examples of such agents include, but are not limited to, phenothiazines, butyrophenones and thioxanthenes.

Anti-emetics are substances which prevent or alleviate nausea or vomiting. An example of such a substance includes, but is not limited to, dramamine.

25 Imaging agents are agents capable of imaging a desired site, e.g. tumor, in vivo. Examples of imaging agents include substances having a label which is detectable in vivo, e.g. antibodies attached to fluorescent labels. The term antibody includes whole antibodies or fragments thereof.

30 Specific targeting agents include agents capable of delivering a therapeutic agent to a desired site, e.g. tumor, and providing a therapeutic effect. Examples of targeting agents include, but are not limited to, agents which can carry toxins or other agents which provide beneficial effects. The targeting agent can be an antibody linked to a toxin, e.g. ricin A or an antibody linked to a drug.

35 Neurotransmitters are substances which are released from a neuron on excitation and travel to either inhibit or excite a target cell. Examples of neurotransmitters

5 include, but are not limited to, dopamine, serotonin, q-aminobutyric acid, norepinephrine, histamine, acetylcholine, and epinephrine.

Cell response modifiers are chemotactic factors such as platelet-derived growth factor (PDGF). Other chemotactic factors include, but are not limited to, neutrophil-activating protein, monocyte chemoattractant protein,
10 macrophage-inflammatory protein, platelet factor, platelet basic protein, and melanoma growth stimulating activity; epidermal growth factor, transforming growth factor (alpha), fibroblast growth factor, platelet-derived endothelial cell growth factor, insulin-like growth factor, nerve growth factor, and bone growth/cartilage-inducing factor (alpha and beta), or other bone morphogenetic protein.

15 Other cell response modifiers are the interleukins, interleukin inhibitors or interleukin receptors, including interleukin 1 through interleukin 10; interferons, including alpha, beta and gamma; hematopoietic factors, including erythropoietin, granulocyte colony stimulating factor, macrophage colony stimulating factor and granulocyte-macrophage colony stimulating factor; tumor necrosis factors, including
20 alpha and beta; transforming growth factors (beta), including beta-1, beta-2, beta-3, inhibin, and activin; and bone morphogenetic proteins.

Uses

Those of ordinary skill in the art will immediately appreciate that the present
25 invention can be utilized in a wide variety of applications to deliver agents into cells. A few particularly preferred applications are discussed in more detail here in order to highlight some of the characteristics and advantages of the inventive systems.

As discussed at length above, the present invention is particularly well adapted for delivery of nucleic acids into cells. As such, the inventive compositions are useful for
30 various applications including gene therapy and antisense regulation. To give but a few examples of particular embodiments of nucleic acid delivery applications of the present invention, inventive compositions can be employed to introduce a gene into specific cells or tissue that will express the protein encoded by that gene and thereby correct a defect caused by a deficiency in that gene in the cells or tissue. Alternatively, inventive
35 compositions can also be used to turn off the function of a specific gene, for example an oncogene in a tumor cell, by delivering antisense messenger RNA into a cell that will bind

5 with the sense messenger RNA so that translation of the message and therefore expression of the protein encoded by that message will not occur.

Inventive compositions can be used in therapeutic gene delivery applications, for example to introduce "suicide genes" into cancer cells that will turn on the cell death pathway. Drug sensitivity genes can also be introduced into tumor cells. For example, 10 cells can be genetically engineered to express prodrug activating enzyme, such as herpes simplex virus thymidine kinase, which phosphorylates ganciclovir creating toxic metabolites that kill tumor cells upon exposure to prodrug.

In the arena of immunotherapy, inventive compositions can be employed in "adoptive immunotherapy" preparations, in which genetically engineered tumor- 15 infiltrating lymphocytes are prepared that express tumor necrosis factor and can be used to treat patients with melanoma. Immunomodulation of tumor cells to invoke an immune response directed toward specific target cell population is yet another area to which this invention can be applied.

Of course, as has already been emphasized, the inventive compositions are not 20 limited in their usefulness to delivery of genes, or even nucleic acids; the compositions can alternatively be used to carry a variety of pharmaceutical compositions. (See Harris, *The Lancet*, 342: 234, 1993).

Examples

25

Example 1: Preparation of Gluconic-Acid-Modified Polyhistidine (Figure 6)

Poly-L-histidine (25 mg, molecular weight range 5,000-15,000, DP = 81) was dissolved in 1mL MES buffer (2-[N-morpholino]ethanesulfonic acid, 25 mM, pH 5.0) to which 17 μ L of an aqueous gluconic acid solution (45% w/v) was added and cooled to 30 4°C. The resulting solution had a final imidazole:gluconic acid mole ratio of 5:1. A solution of EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 776.3 mg, 100 fold molar excess over gluconic acid) and NHS (N-hydroxysuccinimide, 31 mg, NHS:EDAC molar ratio 1:15) was made with 1 mL MES buffer and cooled to 4°C. The EDAC/NHS solution was added to the polyhistidine/gluconic acid solution and allowed to react with 35 stirring at 4°C for 24 hours. The pH of the reaction was brought to 7.0 with NaOH and centrifuged. The supernatant was placed in a Centricon (Amicon, 3000 molecular weight

5 cutoff) and the solvent changed to distilled water by continued centrifugation and water addition. The resulting solution was lyophilized, redissolved in water and further purified using a water phase PD-10 column. The final product was isolated by lyophilization.

10 Example 2: Delivery of Nucleic Acid Encoding β -Galactosidase From a Gluconylated-Polyhistidine/Transferrin-Polylysine Composition

Preparation of cell extract: 5X reporter lysis buffer (purchased from Promega, Madison, WI) was diluted to 1X with water. The cells were then washed with PBS (2ml/well in a 6 well dish) and all of the final wash was removed. 400 μ L of lysis buffer was then added to each well, and the plate was incubated at 37°C for 45 minutes. Cell lysis was confirmed by observation of cells under a microscope. The well was then scraped to dislodge the lysed cells and the lysate was transferred to microfuge tubes with a pipet. The lysate was then vortexed and centrifuged at 14,000 rpm for 2 minutes. The supernatant was collected transferred to fresh tubes. The extract was then stored on ice or frozen at -70°C.

20 *ONPG assay (Spectral photometric assay: o-nitrophenyl- β -D-galactopyranoside):* 2X assay buffer (200 mM sodium phosphate, pH 7.3; 2 mM magnesium chloride; 100 μ M β -mercaptoethanol; 1.33 mg/ml ONPG) was thawed stored on ice. 50 μ L of cellular extract was transferred into the wells of a 96 well plate, each sample in triplicate. 50 μ L of 2X assay buffer was added to each well and incubated at 37°C for 30 to 60 minutes. The reaction was then stopped by adding 150 μ L 1M NaCO₃. The absorbance was then measured at 405 nm on an automatic plate reader.

30 *X-gal staining procedure:* The X-gal reagent was prepared according to standard procedures using the following reagents: 3.3 mM K₄Fe(CN)₆, 3.3 mM K₃Fe(CN)₆, 1 mM MgCl₂, 2 mg/mL X-gal (from 50 mg/mL stock in N,N-dimethylformamide). The cells were washed twice with PBS, 1 mL 0.5 % glutaraldehyde was added to each well and the cells were incubated for 15 minutes at room temperature. The glutaraldehyde was removed and the cells were rinsed gently three times with PBS. The final rinse was then completely removed. 1 mL X-gal solution was added to each well and incubated at 37°C for a time period of at least 2 hours to overnight.

5 *pHis transfection protocol:* 100,000 cells/well were seeded in 6 well tissue culture plates 24 hours prior to transfection. The DNA stock solution was diluted with 30 mM NaOCOCH₃, pH 5 to 50 µg/mL. The concentrations of stock solutions for gluconylated polyhistidine and transferrin-polylysine were 230 µg/mL and 300 µg/mL respectively. The concentrations of gluconylated-polyhistidine and transferrin-polylysine were varied, as shown in Figure 9, in each tube and brought to a final volume of 300 µl with acetate buffer (300 mM sodium acetate, pH 5). The complexes were mixed by adding gluconylated-polyhistidine/transferrin-polylysine solution to DNA such that the final concentration of DNA (pCMV-β-gal) in each well in triplicate was 5 µg. Triplicate wells were provided for each DNA:polyhistidine:transferrin-polylysine ratio tested. The DNA/gluconylated-polyhistidine/transferrin-polylysine transfection solution was incubated for 45 minutes. Meanwhile the cells were washed three times with 2 mL PBS per well. 2.4 mL of Opti-MEM (Gibco, Grand Islands, NY) was added to each of the DNA/gluconylated-polyhistidine/transferrin-polylysine complexes to bring the total volume in the tube to 3.0 mL. 1.0 mL of the complex solution was layered onto each of the triplicate wells such that 5 µg of pCMV-β-gal DNA was delivered to each well and placed in the incubator for 5 hours at which time the transfection medium was removed and replaced with regular growth medium (Dulbecco's modified eagle medium; 10% fetal calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin). Twenty-four hours post-transfection the growth medium was replaced with fresh growth medium. β-galactosidase activity was measured 48 hours post-transfection according to manufacturers instructions (Promega, Madison, WI). Figure 8 shows that the DNA/G-pHis/TfpK complexes transfect approximately 10% of the cells. Figure 9 shows that gluconyl-polyhistidine can effectively substitute for chloroquine in the transfection of cells. Furthermore, Figure 10 reveals that G-pHis is non-toxic to cells in vitro and no significant decrease in cell viability is observed.

30 *Ethidium Bromide Exclusion of G-polyhistidine/DNA complexes:* The final DNA concentration is 1 µM in base pairs. The ethidium bromide molecule:DNA base pair ratio is 1:1. 2.11 µg of pCMV-β-gal DNA was added to 100 µL of 30 mM NaOCOCH₃ (pH 5). A range of 0-20.1 µg gluconylated-polyhistidine was made up in a volume of 100 µL with 30 mM NaOCOCH₃ (pH 5). The gluconylated polyhistidine was added to the DNA

5 solution and incubated 30 minutes at room temperature. 30 mM NaOCOCH₃ (pH 5) and PBS (pH 7.4) buffers were each pre-filtered through 0.22 um syringe filters. Each DNA/gluconylated-polyhistidine (w/w) was diluted 1 to 2.5 mL with NaOCOCH₃ (pH 5) buffer and 1 to 2.5 mL with PBS (pH 7.4). 11.8 µL of 100 µg/mL ethidium bromide was added to each sample in triplicate. 0.8 ml of the DNA/gluconylated-polyhistidine complex
10 in either 30 mM NaOCOCH₃ (pH 5) or PBS (pH 7.4) was loaded to a photon correlation spectrometer (Brookline Instruments Corp.) to measure complex size by light scattering. Figure 11 demonstrates the G-pHis efficiently condenses DNA at pH 5. This procedure was also repeated for the DNA/G-pHis/TfpK complex and the results are shown in Figure 12.

15

Example 3: Assaying DNA Complexation by Cationic Polymers

The ability of the packaging agent to bind DNA can be assessed by monitoring complex formation with DNA using gel electrophoresis. The mobility of DNA on the gel will be retarded by complex formation, and the absence of any mobility of DNA on the
20 gel suggests the complexation of all of the DNA. Preferably, complexation of DNA and the cationic polymer occurs as a ratio of 1:1 DNA/cationic polymer, and most preferably at a ratio of 1:3 DNA/cationic polymer as shown in Figure 13 and 14 for DNA transferrin-polylysine and DNA/G-pHis mixtures. Figure 15 depicts the gel electrophoresis of DNA/p-His mixtures and shows complexation at a weight:weight ratio of 1:0.5 DNA/p-His. Condensing of plasmid DNA can also be monitored by observing the ethidium
25 bromide exclusion. For example, if gluconylated polyhistidine is used as the cationic polymer, the gluconylated polyhistidine efficiently condenses DNA at pH 5 where the gluconylated polyhistidine is ~ 45% protonated. DNA is not condensed as effectively, however, at pH 7.4 where gluconylated polyhistidine is ~ 5% protonated, as shown in
30 Figure 11.

Other embodiments

Those of ordinary skill in the art will appreciate that the foregoing has been a description of certain preferred embodiments of the present invention. This description is
35 not intended to limit the spirit or scope of the present invention, as embodied in the following claims.

5 We claim:

Claims

1. An endosomal lysing system free of polyethyleneimine.
2. The endosomal lysing system of claim 1, comprising a biocompatible compound.
3. The endosomal lysing system of claim 1, comprising a bioresorbable compound.
4. The endosomal lysing system of claim 1, comprising a biocompatible and bioresorbable compound.
5. A biocompatible endosomal lysing system.
6. A bioresorbable endosomal lysing system.
7. A biocompatible and bioresorbable endosomal lysing system.
8. The lysing system of claims 1, 2, 3, 4, 5, 6, or 7, comprised of a molecule containing a plurality of proton acceptor sites.
9. The lysing system of claim 8, wherein the molecule is polycationic at pH 4.
10. The lysing system of claim 8, wherein the molecule is an imidazole containing compound.
11. The lysing system of claim 10, wherein said imidazole containing compound is selected from the group consisting of histidine, histamine, vinylimidazole, polymers thereof, and any combinations of histidine, histamine, vinylimidazole and polymers thereof.
12. The lysing system of claim 8, comprised of a polymeric lysing agent.

- 1 13. The polymeric lysing agent of claim 12, wherein the polymeric lysing agent is
2 combined in a form selected from the group consisting of:
3 mixed polymers;
4 linear co-polymers;
5 branched co-polymers; and
6 dendrimer branched co-polymers.
7
- 8 14. The lysing system of claim 10, wherein said compound is further functionalized
9 with one or more hydrophilic groups.
10
- 11 15. The lysing system of claim 14, wherein said one or more hydrophilic groups is
12 selected from the group consisting of gluconic acid, carbohydrates, nucleic acids, and
13 amino acids.
14
- 15 16. The lysing system of claim 10, wherein said compound is further functionalized
16 with a targeting agent selected from the group consisting of low density lipoproteins,
17 transferrin, asialoglycoproteins, gp120 envelope protein of human immunodeficiency virus,
18 antibodies and carbohydrates.
19
- 20 17. The lysing system of claim 1, 2, 3, 4, 5, 6 or 7, comprised of a biodegradable
21 polymer.
22
- 23 18. The lysing system of claim 17, wherein the biodegradable polymer is a polymer of
24 biomolecules.
25
- 26 19. The lysing system of claim 18, wherein the biomolecules are selected from the
27 group consisting of proteins, amino acids, nucleotides, carbohydrates, and lipids.
28
- 29 20. The lysing system of claim 17, wherein the polymeric lysing agent is selected from
30 the group consisting of:
31 polyhistidine;
32 polyhistidine and polylysine;

1 lysine and polyhistidine;
2 histidine and polylysine;
3 lysine and histidine; and
4 any combinations thereof.
5

6 21. The lysing system of claim 20, wherein said lysing agent is further functionalized
7 with one or more hydrophilic groups.
8

9 22. The lysing system of claim 21, wherein said one or more hydrophilic groups is
10 selected from the group consisting of gluconic acid, carbohydrates, nucleic acids, and
11 amino acids.
12

13 23. The lysing system of claim 20, wherein said lysing agent is further functionalized
14 with a targeting agent selected from the group consisting of low density lipoproteins,
15 transferrin, asialoglycoproteins, gp120 envelope protein of human immunodeficiency virus,
16 antibodies and carbohydrates.
17

18 24. The lysing system of claim 20, wherein said polymeric lysing agent is combined in
19 a form selected from the group consisting of:

20 mixed polymers,
21 linear co-polymers;
22 branched co-polymers; and
23 dendrimer branched co-polymers.
24

25 25. A biocompatible composition comprising:
26 a packaging agent, characterized by an ability to bind to a therapeutic agent and
27 mediate import into endosomes, and;
28 an endosomal lysing agent.
29

30 26. The biocompatible composition of claim 25, wherein said therapeutic agent
31 comprises a nucleic acid.
32

- 1 27. The composition of claim 25 or 26, wherein the packaging agent associates with
2 the therapeutic agent through a covalent interaction.
3
- 4 28. The composition of claim 25 or 26, wherein the packaging agent associates with
5 nucleic acid through a non-covalent interaction.
6
- 7 29. The composition of claim 26, wherein the packaging agent comprises a polycation.
8
- 9 30. The composition of claim 26, wherein the packaging agent condenses the nucleic
10 acid.
11
- 12 31. The composition of claim 26, wherein the packaging agent condenses the nucleic
13 acid to a size less than 150 nm.
14
- 15 32. The composition of claim 26, wherein the packaging agent comprises a material
16 with a high charge density.
17
- 18 33. The composition of claim 26, wherein the packaging agent contains a functionality
19 providing a charge which is present every monomer unit within the structure.
20
- 21 34. The composition of claim 26, wherein the packaging agent is selected from the
22 group consisting of:
23 polylysine;
24 polyhistidine;
25 polylysine and polyhistidine;
26 lysine and polyhistidine;
27 polylysine and histidine; and
28 any combinations thereof.
29
- 30 35. The composition of claim 26, wherein one or both of the packaging agent and the
31 lysing agent is further functionalized with a hydrophilic moiety.
32

1 36. The composition of claim 35, wherein said hydrophilic moiety is selected from the
2 group consisting of gluconic acid, carbohydrates, nucleic acids, and amino acids.

3

4 37. The composition of claim 26, wherein the packaging agent preferably forms a
5 complex with a nucleic acid in a weight:weight ratio of 1:3.

6

7 38. The composition of claim 26, wherein the packaging agent forms a complex with a
8 nucleic acid in a ratio of packaging agent to nucleic acid in the range of 1:3 to 1:10.

9

10 39. The composition of claim 26, wherein one or both of the packaging agent and the
11 lysing agent is covalently linked to a targeting ligand.

12

13 40. The composition of claim 39, wherein the targeting ligand is selected from the
14 group consisting of transferrin, low-density lipoprotein (LDL), asialoglycoproteins, gp120
15 envelope protein of the human immunodeficiency virus (HIV), diphtheria toxin, antibodies,
16 and carbohydrates.

17

18 41. The composition of claim 26, wherein the endosomal-lysing agent contains one or
19 more proton acceptor sites having a pKa in the range of 4 to 7.

20

21 42. The composition of claim 41, wherein the lysing agent is a polycation.

22

23 43. The composition of claim 26, wherein the lysing agent is an imidazole containing
24 compound.

25

26 44. The lysing agent of claim 43, wherein said imidazole containing compound is
27 selected from the group consisting of histidine, histamine, vinylimidazole, polymers
28 thereof, and any combinations of histidine, histamine, vinylimidazole and polymers
29 thereof.

30

31 45. The lysing agent of claim 44, comprised of a polymeric lysing agent.

32

- 1 46. The polymeric lysing agent of claim 45, wherein said polymeric lysing agent is
2 combined in a form selected from the group consisting of:
3 mixed polymers;
4 linear co-polymers;
5 branched co-polymers; and
6 dendrimer branched co-polymers.
7
- 8 47. The composition of claim 26, wherein the lysing agent is a polymer of
9 biomolecules.
10
- 11 48. The composition of claim 47, wherein the biomolecules are selected from the
12 group consisting of proteins, amino acids, nucleotides, carbohydrates, and lipids.
13
- 14 49. The composition of claim 26, wherein the packaging agent and lysing agent
15 comprise the same material.
16
- 17 50. The composition of claim 26, wherein the packaging agent and lysing agent
18 comprise two or more materials.
19
- 20 51. The composition of claim 26, wherein the polymeric lysing agent is selected from
21 the group consisting of:
22 polyhistidine;
23 polyhistidine and polylysine;
24 lysine and polyhistidine;
25 histidine and polylysine;
26 lysine and histidine; and
27 any combinations thereof.
28
- 29 52. The lysing system of claim 51, wherein said polymeric lysing agent is combined in
30 a form selected from the group consisting of:
31 mixed polymers,
32 linear co-polymers;

1 branched co-polymers; and
2 dendrimer branched co-polymers.

3

4 53. A biocompatible and bioresorbable composition comprising:
5 a nucleic acid binding agent; and
6 an endosomal lysing agent that is polycationic at pH 4 and that has a plurality of
7 proton acceptor sites each having a pKa in the range of 4 to 7, the nucleic acid binding
8 agent and endosomal lysing agent being selected so that, when the composition is
9 combined with DNA and eukaryotic cells, the composition mediates introduction of the
10 DNA into an endosome and then lyses the endosome in the absence of a known
11 endosomolytic agent.

12

13 54. The composition of claim 53 wherein the known endosomolytic agent is one or
14 more of chloroquine, fusogenic peptides, inactivated adenoviruses, and polyethyleneimine.

15

16

17 55. A method of lysing an endosome, the method comprising the steps of:
18 providing a composition for endosomal uptake into the cell; and
19 contacting the composition with the cell in the presence of an endosomal lysing
20 agent, wherein said endosomal lysing agent is selected from the group consisting of:
21 an endosomal lysing agent free of polyethyleneimine;
22 a biocompatible endosomal lysing system free of polyethyleneimine;
23 a bioresorbable endosomal lysing system free of polyethyleneimine;
24 a biocompatible and bioresorbable endosomal lysing system free of
25 polyethyleneimine;
26 a biocompatible endosomal lysing system;
27 a bioresorbable endosomal lysing system; and
28 a biocompatible and bioresorbable endosomal lysing system.

29

30 56. The method of claim 55, wherein said composition comprises a therapeutic agent,
31 wherein said therapeutic agent is bound covalently or non-covalently to a delivery agent,

1 and wherein said endosomal lysing agent comprises a compound having a plurality of
2 proton acceptor sites; and
3 wherein said endosomal lysing agent is covalently or non-covalently associated
4 with the composition.
5

6 57. The method of claim 56, wherein the plurality of proton acceptor sites of said
7 endosomal lysing agent have pKa's within the range of 4 to 7, and wherein said
8 endosomal lysing agent is polycationic at pH 4.
9

10 58. The method of claim 56, wherein said endosomal lysing agent comprises a
11 polymer of biomolecules.
12

13 59. The method of claim 56, wherein the lysing agent is an imidazole containing
14 compound.
15

16 60. The method of claim 59, wherein said imidazole containing compound is selected
17 from the group consisting of histidine, histamine, vinylimidazole, polymers thereof, and
18 any combinations of histidine, histamine, vinylimidazole and polymers thereof.
19

20 61. The method of claim 60, wherein said lysing agent is comprised of a polymeric
21 lysing agent.
22

23 62. The polymeric lysing agent of claim 61, wherein said polymeric lysing agent is
24 combined in a form selected from the group consisting of:

25 mixed polymers;

26 linear co-polymers;

27 branched co-polymers; and

28 dendrimer branched co-polymers.
29

30 63. The lysing system of claim 56, wherein said lysing agent is further functionalized
31 with one or more hydrophilic groups.
32

1 64. The lysing system of claim 63, wherein said one or more hydrophilic groups is
2 selected from the group consisting of gluconic acid, carbohydrates, nucleic acids, and
3 amino acids.

4
5 65. The lysing system of claim 56, wherein said lysing agent is further functionized
6 with a targeting agent selected from the group consisting of low density lipoproteins,
7 transferrin, asialoglycoproteins, gp120 envelope protein of human immunodeficiency virus,
8 antibodies and carbohydrates.

9
10 66. The method of claim 58, wherein the biomolecules are selected from the group
11 consisting of proteins, amino acids, nucleotides, carbohydrates and lipids.

12
13 67. The method of claim 56, wherein the endosomal lysing agent is selected from the
14 group consisting of:

15 polyhistidine;
16 polyhistidine and polylysine;
17 lysine and polyhistidine;
18 histidine and polylysine;
19 lysine and polyhistidine; and
20 any combinations thereof.

21
22 68. The method of claim 67, wherein said lysing agent is combined in a form selected
23 from the group consisting of:

24 mixed polymers;
25 linear co-polymers;
26 branched co-polymers; and
27 dendrimer branched co-polymers.

28
29 69. The method of claim 56, wherein said therapeutic agent comprises a nucleic acid.

30
31 70. The method of claim 69, wherein the packaging agent comprises a polycation.

32

- 1 71. The method of claim 69, wherein the packaging agent condenses the nucleic acid.
2
- 3 72. The method of claim 69, wherein the packaging agent condenses the nucleic acid
4 to a size less than 150 nm.
5
- 6 73. The method of claim 69, wherein the packaging agent comprises a material with
7 high charge density.
8
- 9 74. The method of claim 69, wherein the packaging agent contains a functionality
10 providing a charge which is present every monomer unit within the structure.
11
- 12 75. The method of claim 69, wherein the packaging agent is selected from the group
13 consisting of:
14 polylysine;
15 polyhistidine;
16 polylysine and polyhistidine;
17 lysine and polyhistidine;
18 polylysine and histidine; and
19 any combinations thereof.
20
- 21 76. The method of claim 69, wherein one or both of the packaging agent and the lysing
22 agent is further functionalized with a hydrophilic moiety.
23
- 24 77. The method of claim 69, wherein said hydrophilic moiety is selected from the
25 group consisting of gluconic acid, carbohydrates, nucleic acids, and amino acids.
26
- 27 78. The method of claim 69, wherein the packaging agent preferably forms a complex
28 with a nucleic acid in a weight:weight ratio of 1:3.
29
- 30 79. The method of claim 69, wherein the packaging agent forms a complex with a
31 nucleic acid in a ratio of packaging agent to nucleic acid in the range of 1:3 to 1:10.
32

1 80. The method of claim 69, wherein one or both of the packaging agent and the lysing
2 agent is covalently linked to a targeting ligand.

3
4 81. The method of claim 80, wherein the targeting ligand is selected from the group
5 consisting of transferrin, low-density lipoprotein (LDL), asiaglycoproteins, gp120
6 envelope protein of human immunodeficiency virus (HIV), diphtheria toxin, antibodies, and
7 carbohydrates.

8
9 82. A method for introducing nucleic acids into a cell or a subcellular component, the
10 method comprising steps of:

11 providing a bioresorbable and biocompatible delivery composition comprising:

12 a nucleic acid binding component;

13 an endosomal lysing component comprising a plurality of proton acceptor
14 sites having pKas within the range of 4 to 7, which endosomal lysing component is
15 polycationic at pH 4; and

16 a nucleic acid; and

17 contacting the composition with cells in the absence of a known endosomal lysing
18 component selected from the group consisting of chloroquine, polyethyleneimine,
19 fusogenic peptides, inactivated adenoviruses and combinations thereof.

20
21 83. The method of claim 82, wherein the endosomolytic lysing component comprises a
22 polymer of biomolecules.

23
24 84. The method of claim 82, wherein the lysing component is an imidazole containing
25 compound.

26
27 85. The method of claim 84, wherein said imidazole containing compound is selected
28 from the group consisting of histidine, histamine, vinylimidazole, polymers thereof, and
29 any combinations of histidine, histamine, vinylimidazole and polymers thereof.

30
31 86. The method of claim 82, wherein said endosomal lysing component is further
32 functionalized with one or more hydrophilic groups.

1 87. The method of claim 86, wherein said one or more hydrophilic groups is selected
2 from the group consisting of gluconic acid, carbohydrates, nucleic acids, and amino acids.

3
4 88. The method of claim 82, wherein said endosomal lysing component is further
5 functionized with a targeting agent selected from the group consisting of low density
6 lipoproteins, transferrin, asialoglycoproteins, gp120 envelope protein of human
7 immunodeficiency virus, antibodies and carbohydrates.

8
9 89. A non-immunogenic artificial virus less than 150 nM in size, comprising:
10 a nucleic acid packaging agent;
11 an endosomal lysing component comprising a plurality of proton acceptor
12 sites having pKas within the range of 4 to 7, which endosomal lysing component is
13 polycationic at pH 4; and
14 a nucleic acid.

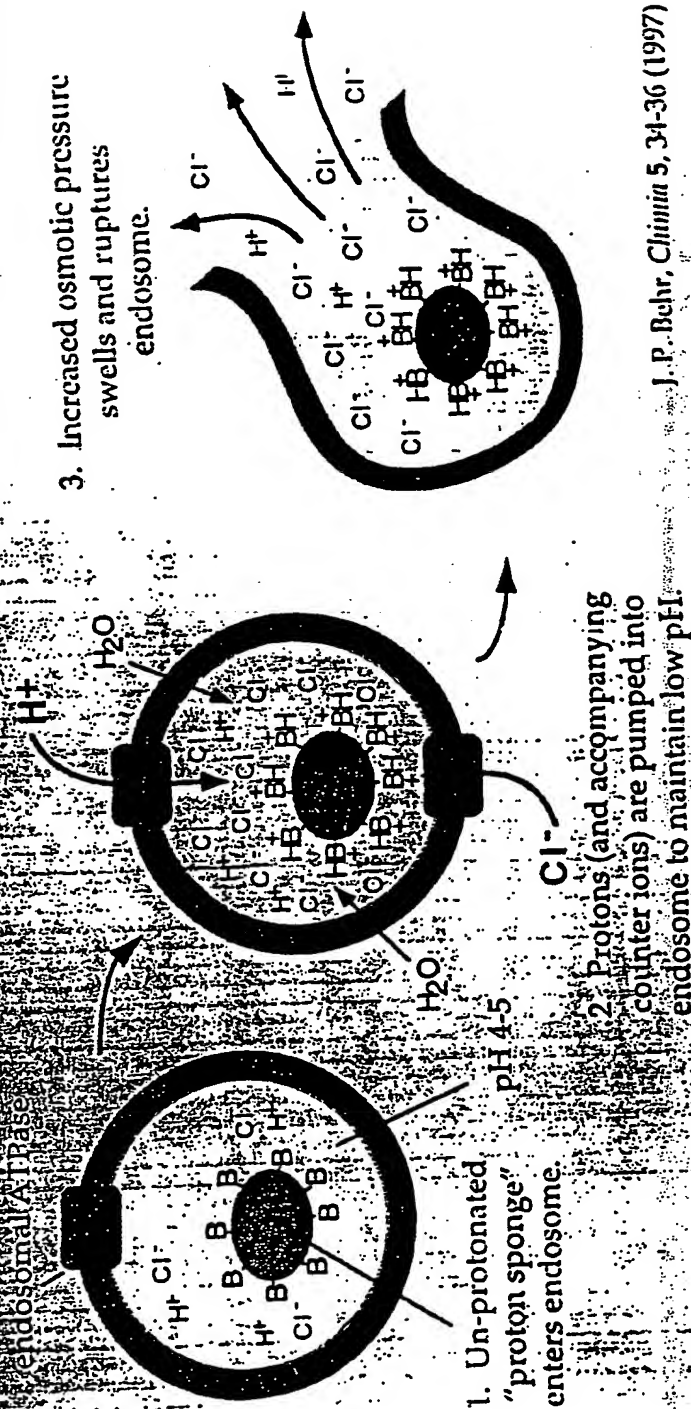
15
16 90. The artificial virus of claim 89, wherein the endosomal lysing component
17 comprises a polymer of biomolecules.

18
19 91. A cell delivery composition comprising:
20 a compound to be delivered to a cell;
21 a delivery agent bound to compound; and
22 an endosomolytic agent comprising a plurality of proton acceptor sites having
23 pKas within the range of 4 to 7, which endosomal lysing component is polycationic at pH
24 4, the endosomolytic agent being covalently or non-covalently associated with the
25 compound or delivery agent.

26
27 92. The cell delivery composition of claim 91, wherein the compound to be delivered
28 to a cell is selected from the group consisting of anti-AIDS substances, anti-cancer
29 substances, antibiotics, immunosuppressants, anti-viral substances, enzyme inhibitors,
30 neurotoxins, opioids, hypnotics, antihistamines, lubricants, tranquilizers, anti-convulsants,
31 muscle relaxants, anti-Parkinson substances, anti-spasmodics and muscle contractants,
32 miotics, anti-cholinergics, anti-glaucoma compounds, anti-parasite compounds, anti-

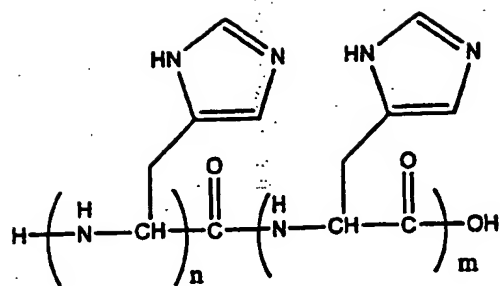
- 1 protozoal compounds, anti-hypertensives, analgesics, anti-pyretics, anti-inflammatory
- 2 agents, local anesthetics, ophthalmics, prostaglandins, anti-depressants, anti-psychotic
- 3 substances, anti-emetics, imaging agents, specific targeting agents, neurotransmitters,
- 4 proteins, cell response modifiers, vaccines, anti-sense agents, RNA, and ribozymes.

"Proton-sponge" polymers are believed to mediate release of DNA from lysosomes.



J. P. Behr, *Chemia* 5, 34-36 (1997)

Figure 2



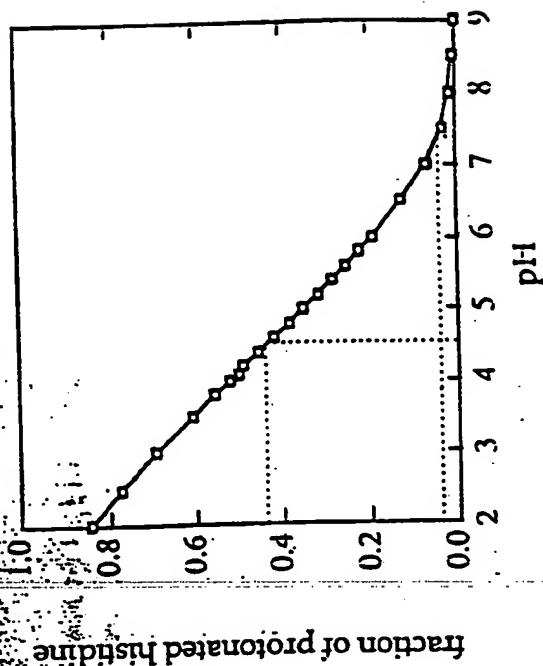
Polyhistidine
Figure 3

4/15

may function as a proton sponge.



polyhistidine
(pHis)



Wang and Huang, Biochemistry 23,
4409-4416 (1984)

Figure 4

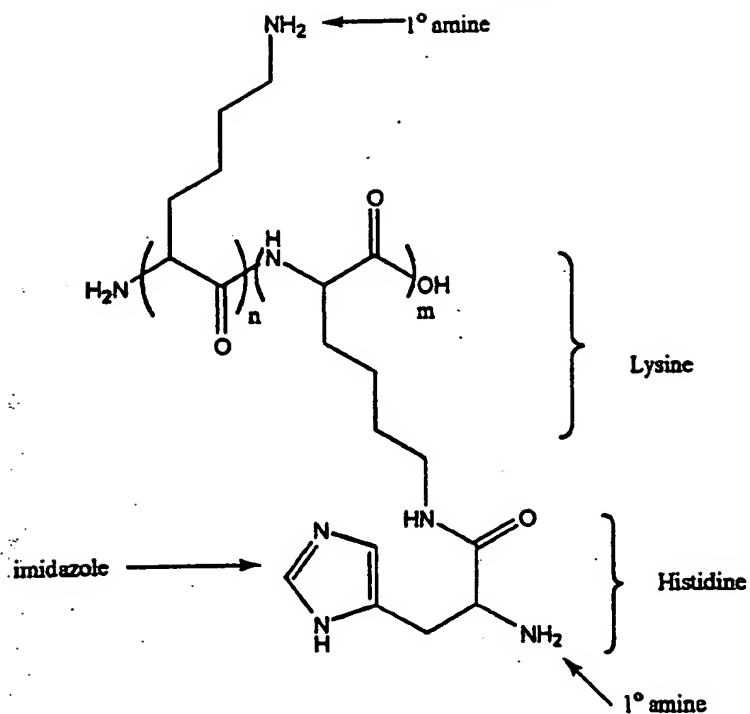


Figure 5A: Copolymer of Polylysine and Histidine

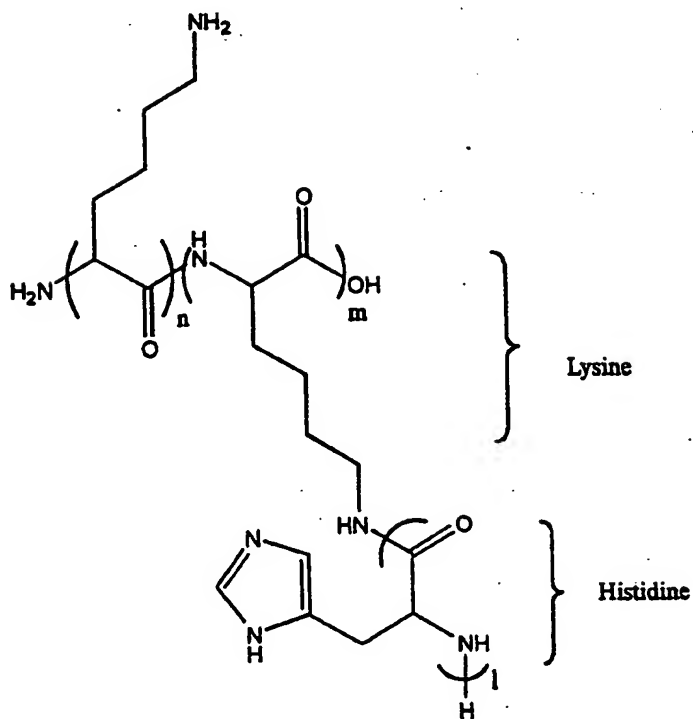


Figure 5B: Copolymer of Polylysine and Polynisidine

Derivatization of polyhistidine with gluconic acid results in improved solubility.

- Addition of hydrophilic hydroxyls is expected to increase solubility of polymer.
- Approximately 10-20% of the imidazoles are substituted with gluconoyl moiety.
- Gluconoyl polyhistidine is soluble at neutral pH.

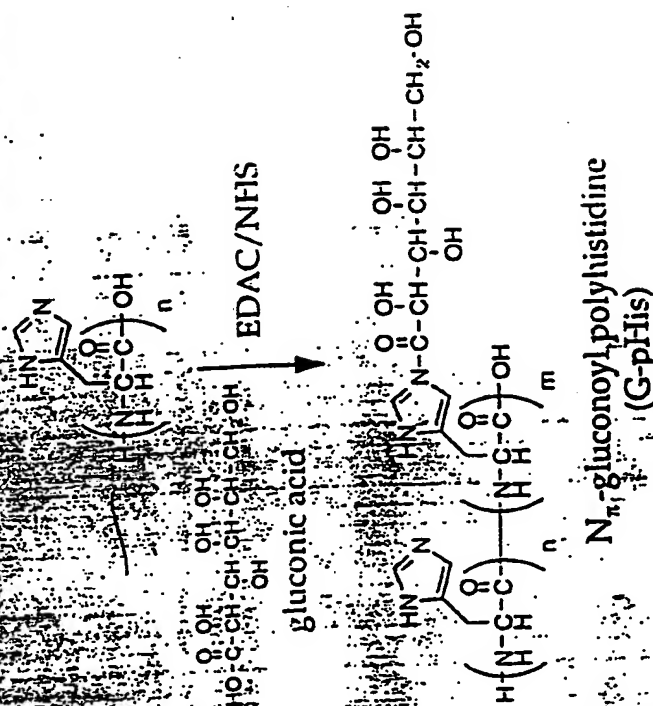
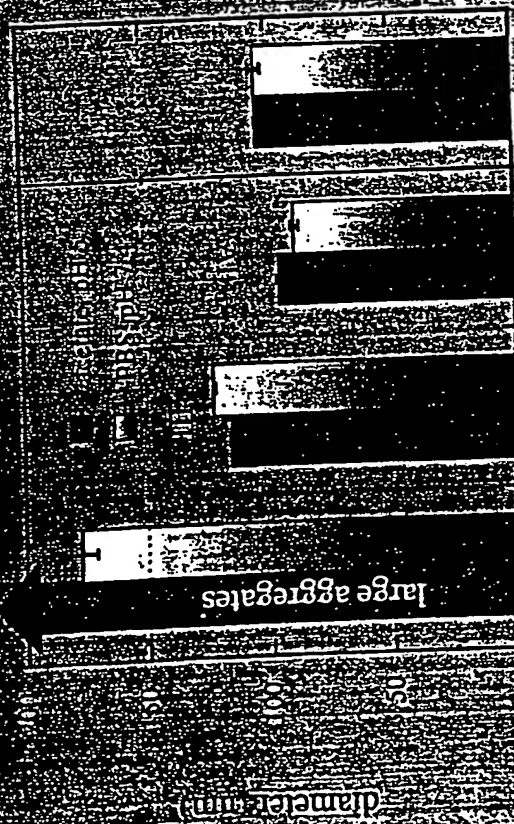


Figure 6

7/15

are well below the size

of the complexes are well below the size



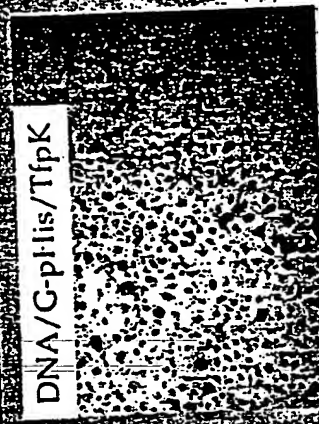
• DNA/TfPK form complexes ~100 nm in diameter.

• DNA/G-pHis/TfPK complexes are of similar size.

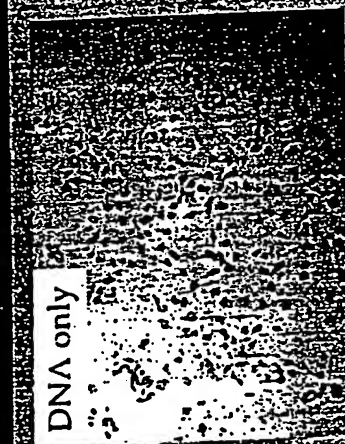
• These complexes appear to have the physical requirements for mediating transfection.

Figure 7

LDV-3000 transfection complexes transfected
approximately 10% of COS-7 cells.



A DNA only



B LipofectAMINE



C DNA/G-pHis/TspK

X-gal staining of COS-7 cells 48 h post-transfection:

- A. DNA only: no blue cells
- B. DNA/G-pHis/TspK: ~10% (13/15)
- C. LipofectAMINE: ~45%

Figure 9

DNA/G-pHis/TlpK complexes effectively transfect COS-7 cells in culture.

- Chloroquine is lysosomolytic; most likely aids in release of DNA to cytoplasm.
- Transferrin-polylysine complexes require chloroquine (Chl) for transfection.
- Gluconoyl-polyhistidine can effectively substitute for chloroquine.

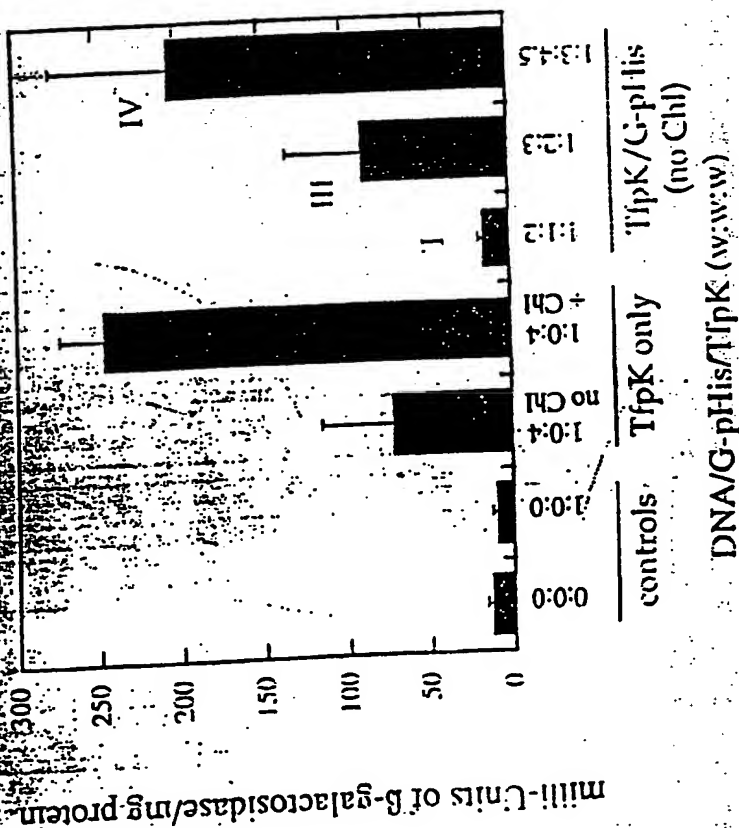


figure 9

10/15

G-pHis is non-toxic to COS-7 cells *in vitro*.

- MTT assay reflects proliferation of cells in culture.
- No significant decrease in cell viability is observed even at 20 $\mu\text{g/ml}$ G-pHis.
- Typical concentration used in transfection is 10 $\mu\text{g/ml}$.

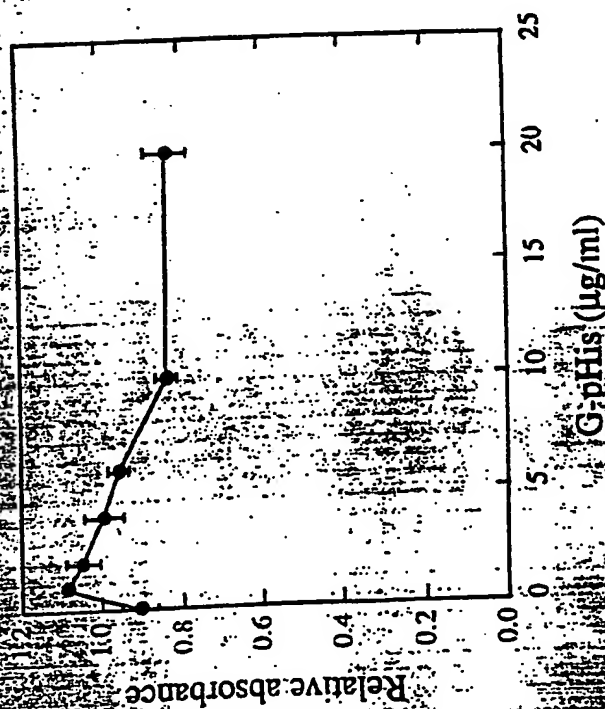
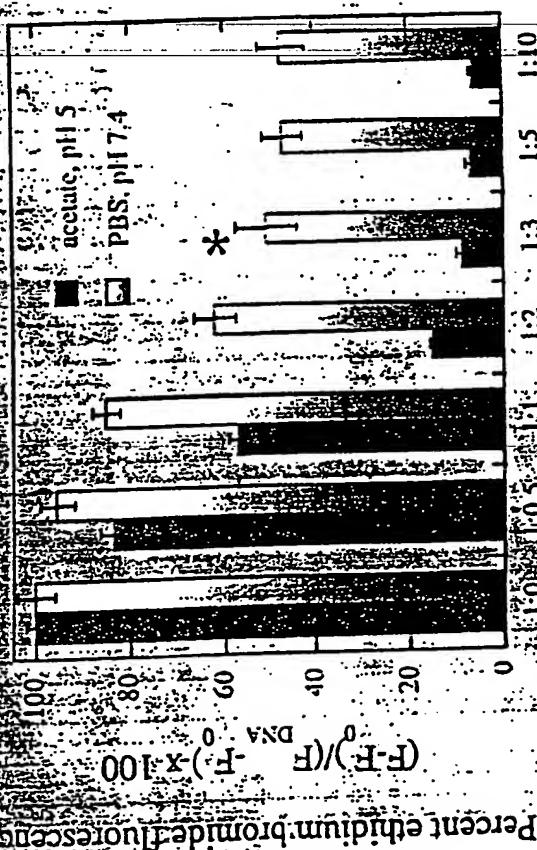


figure 10

11/15

G-pHis condenses plasmid DNA as revealed by ethidium bromide exclusion



DNA/G-pHis (w:w)

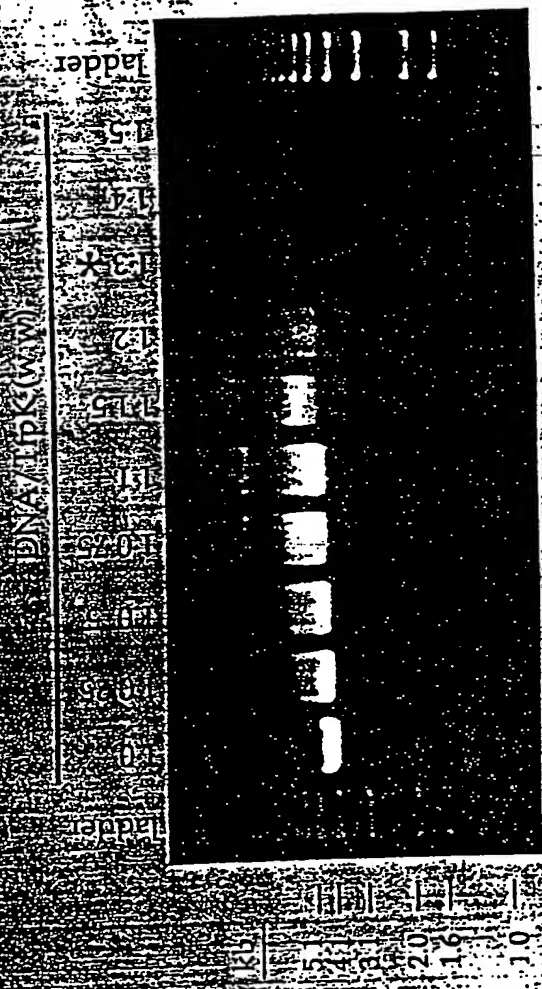
(Complexes formed at pH 5; then diluted with either acetate (pH 5) or PBS (pH 7.4).)

- G-pHis efficiently condenses DNA at pH 5 (G-pHis is ~45% protonated).
- DNA is less condensed at pH 7.4 (G-pHis is ~5% protonated).

Figure 11

Cellulose transferin polylysine

complex formation



- TfpK retards mobility of DNA on gel.
- All DNA is complexed at DNA/TfpK = 1:3 (w:w).

***Charge-neutral complex**

Figure 13

- G-pHis retards mobility of DNA on gel.
- All DNA is complexed at DNA/G-plis 1:3 (w:w).



* Charge-neutral complex

Figure 14

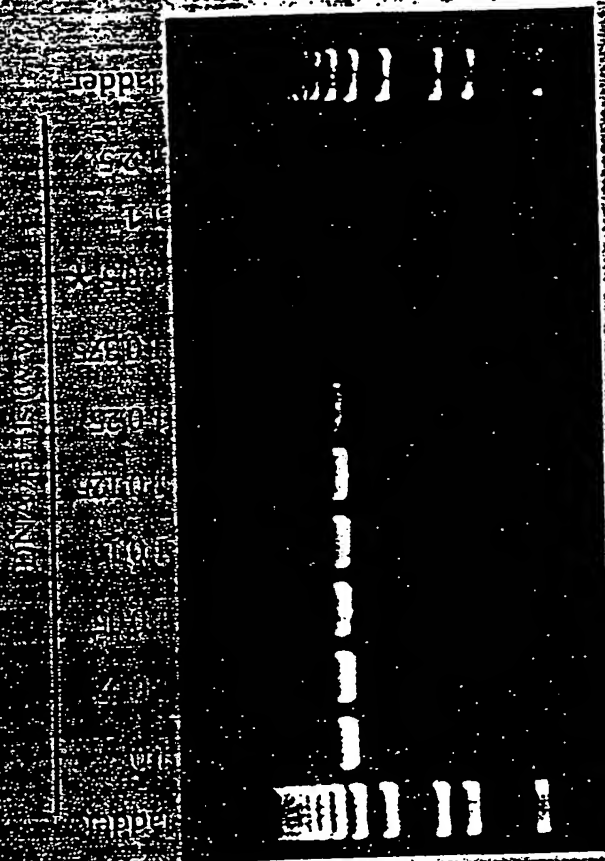
Electrophoretic mobility of DNA/pHis complexes reveals

(complex formation)

- pHis retards mobility of DNA on gel

- All DNA is complexed at DNA/pHis = 1:0.5 (w:w).

- The first complex which is completely retarded is considered to be charge-neutral.



* Charge-neutral complex

Figure 15